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BIOLOGICAL EVALUATION OF RADIOPROTECTIVE DRUGS

FINAL REPORT

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The cellular and molecular mechanisms of radioprotection by aminothiols have been investigated through the use of cultured mammalian (CHO) cells and a whole-animal model, specific-pathogen free mice. For each of these systems, detailed structure-activity relationships were determined relating the effects of the different drugs on various endpoints for the survival of clonogenic cells and on the induction and repair of several types of DNA lesions in these same cells. The data with cultured cells have been used as a means of understanding or predicting the effects of the phosphate-blocked derivatives of these thiols on tissues. The major finding from the in vitro studies is the fact that the modulation of cell survival and DNA double-strand break induction correlates closely for a series of structurally-different thiols and aminothiols. The in vivo studies suggest that tissue oxygenation plays an important role in determining both the extent and mechanism of protection, and that understanding the interrelationships between oxygen, aminothiols and radiation sensitivity will be a crucial step in our ability to utilize such drugs effectively.

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SUMMARY

This final report summarizes our studies of the mechanism(s) of action of radioprotective aminothiols. Using cultured Chinese hamster ovary (CHO) cells we have accumulated detailed structure-activity relationships describing the relative effects of a series of thiols on cell survival, DNA single-strand break (SSB) induction and repair, DNA double-strand break (DSB) induction and repair, and on the induction of chromosome aberrations. Because of the considerable current interest in the measurement of DSBs we have studied DSB induction in some detail. During the course of this contract, five compounds were characterized using these various assays: dithiothreitol, cysteamine, WR-1065, WR-255591 and WR-151326. Based on these data we have been able to make some conclusions about the relationship between structural features and protective mechanism(s). The most intriguing observation is that the molecular processes underlying radioprotection vary markedly with the changes in thiol structure, suggesting in turn that there is no single "mechanism" of protection. Each of the drugs had a differential effect on the induction of different classes of DNA lesion, and this behavior varied among the drugs. However, of these various lesions, the induction of DSBs appears to closely predict the resulting effect of a drug on cell survival independently of the thiol structure. Studies have also been performed to evaluate the relationships between DNA SSB induction, clonogenic stem-cell survival, and loss of tissue function in mouse tissues irradiated in vivo. We characterized two compounds in detail--WR-2721 and WR-3689--for their effects on DNA damage and clonogenic cell survival in bone marrow and jejunum. The effects on clonogenic cell survival correlated closely with the effects of the drugs on the survival of the animals. The results with the DNA damage assay suggest an important role for oxygen in modifying the relationships between DNA-level effects and the biological effects of the radiation. They also suggest that the development of sensitive assays for DSB induction and repair in these tissues, perhaps by an extension of the neutral elution assay, would be an important step to better understanding how well these in vitro relationships can be extrapolated to the in vivo situation.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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The studies performed under contract DAMD 17-86-C-6105 were designed to evaluate those factors that govern the radioprotective ability of various thiols and aminothiols. As with any long-term study, several of the specific aims evolved along with the project itself. The original proposal could be divided into 2 basic sections: (a) studies with cultured cells, and (b) studies with animal tissues. These two subsections will be discussed in turn. At the end of this document, I will try to summarize how the in vitro data have been used to interpret the more complex in vivo data.

A. STUDIES WITH CULTURED CHO CELLS

The basic premise here was to collect and correlate data for a series of structurally-related compounds with respect to their relative effect on cell survival and on the induction and repair of various types of DNA damage.

A1. Cell Survival

The first step in all of our studies was to measure the effect of the particular drug on cell survival using a clonogenic assay in which survival is defined as the ability of a cell to proliferate indefinitely. This is an extremely reproducible assay and can be used to optimize conditions with respect to such variables as drug dosage and timing. The drugs that we evaluated and their structures are shown in Table I.

The general approach in evaluating any new compound was as follows:

i) First, we determined the effect of a 30 min pretreatment with various concentrations of each drug on the surviving fraction (SF) after a single dose (10 Gy) of γ-rays. Results are shown in Fig. 1A for the 5 drugs that were studied. There was clearly a great difference in the concentration of each drug required to produce a given level of protection.

ii) Based on these data, we selected a concentration of each drug that would give a similar degree of protection but that was not toxic to the cells. A protection factor (PF) of between 2 and 2.5 was deemed optimal; the concentrations chosen were 4 mM WR-1065, 6 mM WR-255591, 6 mM WR-151326, 10 mM cysteamine, and 25 mM dithiothreitol (DTT). The time course of protection by each of these thiols was then determined. None of the drugs had any efficacy when given after irradiation. The time-dependence of pretreatment is shown in Fig. 1B. Clearly, the various thiols varied greatly with respect to the rate at which protection was achieved, with DTT and cysteamine giving maximal protection within minutes and the two more structurally complex aminothiols being much slower.

iii) For the purpose of obtaining quantitative PF values, complete radiation dose-response curves were determined for a 30-

min pretreatment with the above-mentioned concentrations of each compound. The data for WR-1065 (1) and WR-255591 (2) have been published in some detail. Fig. 2 shows similar data for cysteamine. Several generalizations can be made on the basis of these data:

a) For each drug, the PF appeared to be independent of the dose of radiation, i.e. the drugs were radiation dose-modifying. The survival curves for each drug were rigorously analyzed using either the linear-quadratic (LQ) model or the multi-target (MT) model. The parameters of these 2 models (LQ: α and β; MT: D_0, D_Q, n) all behaved in a dose-modifying way, as discussed in detail for WR-1065 (1).

b) The PF for each drug was identical whether the irradiation was given at 37°C or on ice.

A2. Single-strand Break Induction

The technology of choice here was alkaline elution (3) because of its extreme sensitivity and because we had already demonstrated that the technique could be suitably adapted to mouse tissues (4,5). This latter factor would be important in view of our goal of extending these relationships to tissues irradiated in vivo (section B).

The various drugs were evaluated in turn for their ability to protect CHO cells from SSB induction under exactly the same conditions that the above survival measurements were made. Table II compares the PF values for SSB induction (PF[SSB]) and cell survival (PF[SF]) for each drug. The PF[SSB] values were obtained from complete dose-response curves for SSB-induction as published for both WR-1065 (1) and WR-255591 (2). It is readily apparent that, for all 5 drugs, the PF[SSB] was much lower than the PF[SF], this being independent of the radiation dose used. In Table II, all of the measurements were made on cells that were chilled on ice at the end of the thiol treatment, i.e., before irradiation. We therefore examined what the situation would be in cells irradiated at 37°C in the presence of different concentrations of each thiol. These data are illustrated in Fig. 3 for the three more complex aminothiols. It is apparent that, while protection increased with drug concentration, the PF[SSB] was always low relative to the PF[SF] under all conditions.

In a publication submitted to a recent conference on "Anticarcinogenesis and Radiation Protection" we plotted these data in a different way (6); the PF[SSB] was plotted versus the corresponding PF[SF], the variable being the concentration of the thiol. These data are reproduced in Fig. 4 and confirm that, for each thiol, protection against SSB induction was always relatively small although different for different thiols. Another informative way of representing such data was discussed by Radford (7,8) who suggested that a plot of -ln(SF) versus SSB frequency should be linear if these lesions correlate with survival; more importantly, the plots for irradiation with or

without various thiols should fall along a common line (i.e., have a common slope) if the modification of SSBs and survival are equivalent. Such a correlation was not observed when we analyzed our data in this manner (data not shown), as was also found for cysteamine (7). This led Radford (7) to conclude that SSBs were not lethal lesions, although, as discussed below, there are other alternative explanations for this lack of correlation since SSB measurements are extremely prone to artifactual sources of error!

However, this form of data analysis presentation is still not optimal; as will be discussed in section A2 for the double-strand break (DSB) induction data, this method can be made even more powerful by using the replicate-plating dual-label method (7,8) although the extent of the lack of correlation between SF and SSB induction that we observed was so dramatic that this is clearly not the explanation for the present results and conclusions.

QUESTION: What are the possible reasons for the relatively poor protection against SSB induction, i.e., why is PF[SSB] << PF[SF]? There are several possible explanations, which we shall consider in turn.

i) It is possible that the concentration of drug oxidation products - H_2O_2 or O_2^- - may be enhanced in irradiated cells, and these could then damage the DNA and increase the level of SSBs selectively in the drug-treated cells, thereby lowering the measured PF[SSB]. However, this possibility is unlikely based on the fact that including catalase (to remove H_2O_2) and/or desferal (to remove iron) had little effect on the PF[SSB] value (1).

ii) As discussed by Quintiliani (9) there is the possibility that either thiyl ($RS^\bullet$) or sulfur peroxyl ($RSO_2^\bullet$) radicals may be produced in irradiated, drug treated cells, and these radicals may then react with DNA to cause SSBs. As discussed above, this would have the effect of lowering the PF[SSB]. This is a reasonably likely event since $RS^\bullet$ radicals are produced in virtually all reactions of RSH with radiation products, and these radicals will react rapidly with O_2 to produce $RSO_2^\bullet$ radicals. The extent of the involvement of these radicals cannot be ascertained at present.

iii) It is widely believed that DSBs correlate closely with cell survival. Nonetheless, it has commonly been assumed that, if thiols protect cells by scavenging $OH^\bullet$ radicals, by H-atom donation, or by inducing anoxia, they would be effective protectors against SSB induction -- even if SSBs were not lethal. However, in section A3 we will describe a simple model based on the Alper Howard-Flanders fixation-repair model that will predict that if the PF[SF] and PF[DSB] values are similar, as indeed we find, then the PF[SSB] should theoretically be much lower than either the PF[SF] or PF[DSB], as again we do find.

A3. Double-strand break induction

Our measurements of DSB induction illustrate well the earlier comment about the evolution of these studies. In our initial studies we found that, when we used the modification of the neutral elution methodology originally described by Bradley and Kohn (10), we obtained a linear DSB-induction curve when we used a pH of 7.0 for all of the solutions, whereas at pH 9.6 the DSB-induction curves were curvilinear. This suggested that the pH 9.6 assay may still be detecting a large component of DSBs arising from overlapping SSBs and/or base damage. Measurements of DSB rejoining appeared to substantiate this argument. Our initial study with WR-1065 (1) therefore used only the pH 7 assay even though this was less sensitive and required the use of supralethal doses of radiation. From these linear dose-responses we obtained a PF[DSB] of ~1.65 for 4mM WR-1065, which was closer to the PF[SF] than was the PF[SSB], but still not equal. This appeared to contrast with the data of Radford (7,8) showing that cysteamine modified survival and pH 9.6 DSB induction equally. We therefore examined a second drug, WR-255591 (2). However, this time we used both the pH 7 and pH 9.6 versions of the neutral elution technique and obtained the results shown in Fig. 5. At both pH's the PF[DSB] was ~ 1.8, i.e., close to, but not identical to, the PF[SF] value of 2.3 for this drug at a concentration of 6 mM. For completeness, we went back and evaluated 4 mM WR-1065 using the pH 9.6 assay and found essentially the same thing, as shown in Table II. Thus, for both drugs, we found that:

$$PF[SF] > PF[DSB, pH\ 7] \sim PF[DSB, pH\ 9.6] \gg PF[SSB]$$

Indeed, when we examined several other drugs, this general trend appeared to be substantiated (Table II).

We therefore asked the question: Why is the PF[DSB] lower than the PF[SF]? There were several possibilities.

i) The drugs could be affecting DSB repair, presumably by enhancing these processes. However, as discussed later in section A.5, we could find no evidence for any such effect, although there are some additional questions (discussed later) about the significance of such measurements.

ii) The drugs could be affecting recovery from potentially lethal damage (PLD). In fact, based on our current knowledge of aminothiols, there are several possible mechanisms by which PLD could be affected. Most notably, Grdina and Nagy (11) had shown that 4 mM WR-1065 significantly inhibited the cell-cycle progression of V79 cells, and suggested that this may allow additional time for recovery from PLD. Our own studies on the involvment of PLD in radioprotection are discussed in section A.6; although our studies have shown that postirradiation treatment of the cells with hypertonic saline, a procedure that reduces the influence of some types of PLD on cell survival, lowered the PF[SF] for WR-1065 to a value similar to the PF[DSB],

our subsequent studies (described below) have led us to re-evaluate the implication of these observations.

iii) The low sensitivity of the pH 7 neutral elution technique meant that we had started out by using radiation doses much higher than those used in the survival studies. It was therefore possible that the PF[DSB] may be dose-dependent. However, at first glance, this seemed unlikely since the pH 7 dose responses were linear with or without the drug and would have to behave very strangely at low radiation doses to give a greater PF[DSB]. What caused us to re-evaluate this conclusion was a series of debates at scientific meetings about the relative merits of various types of neutral elution assay. This led Dr. Jonn Ward and his colleagues at the University of California at San Diego to examine the "DNA" eluting from the filter at pH 7 using electron microscopy. They (personal communication) found that the DNA was not eluting independently of cellular proteins, despite previous claims that SDS proteinase K effectively removed cellular proteins under these conditions. The key factor here appears to be the magnitude of deproteinization, and indeed, a careful search of the literature revealed that at pH 7 proteinase K has a much reduced activity (12).

The dose-response curves for DSB induction at pH 9.6 were non-linear, however, so it was more difficult to rule out the possibility that the PF may increase in the lower-dose range. We therefore adapted our pH 9.6 procedure again, this time by using a modified version of the assay described by Radford (7,8). Briefly, in this assay, the cells are labeled with ^{14}C-TdR, treated, irradiated, and trypsinized. The doses used for control cells are up to 10 Gy while the thiol-treated cells receive up to 30 Gy, as appropriate. The cells are then split into 2 fractions. One of these fractions is plated for cell survival measurements. The other fraction is admixed with ^{3}H-TdR-labeled internal reference cells that have received 30 Gy of x-rays on ice. The cells are then eluted at pH 9.6 as usual. Triplicate elution samples are run for each dose point. The eluted DNA is counted using a dual-label program so that the elution of the ^{14}C-labeled cells and the ^{3}H-labeled internal reference cells can be plotted separately. The level of DSB induction is then calculated as described by Wlodek and Hittelman (13). Because of potential complications with dual-label counting we rigorously compared the data with data obtained using only the ^{14}C-labeled cells, i.e., without the ^{3}H-labeled internal reference cells. We found that the results were identical and just as reproducible using the single-label method, so it was adopted exclusively for the remainder of these studies.

The power of this "replicate plating" technique is derived from the fact that the level of DSBs and survival are determined on exactly the same sample of cells. This enables the construction of what have come to be known as "lethal lesion plots" where the level of DSB induction (averaged among the triplicate samples) for a given sample of cells is plotted

against the negative log (-ln) of the SF measured from triplicate samples of these very same cells. Typical data obtained with this technique are shown for three groups of cells in Fig. 6: (a) control cells receiving only γ-rays; (b) cells pretreated with 6 mM WR-255591; and (c) cells pretreated with 10 mM cysteamine. It is clear that, for all three treatment groups, the data can be fitted by a common slope. The implication of this observation is simple: the DSBs induced in all three groups of cells have an equal "lethal efficiency". This is equivalent to the statement that, over the radiation dose range where cell survival is measured, both WR-255591 and cysteamine protect equally against DSB induction and cell killing. This statement is obviously in conflict with the results based on our earlier high-dose DSB data (Table II) and with similar results obtained by Sigdestad et al. (14), but are in complete agreement with the data of Radford (7,8) for cysteamine which were obtained using the replicate plating method. Why this discrepancy? The answer could be one of two things.

a) It could be related to the fact that, if the lethal lesion plot method is not used, one has to mathematically calculate a PF[SF] value from a pair of survival curves and a PF[DSB] value from a pair of DSB induction curves. This requires some judgement and quickly becomes complicated if the survival or DSB induction curves are not easy to describe mathematically or if the treatment is NOT dose-modifying for either end-point. In any case, there are a lot of potential sources of error in this method. The involvment of this potential artifact can be gauged by re-evaluating the low-dose data used for the lethal lesion plots (Fig. 6) by the alternative method, as shown in Fig. 7. The degree of DSB induction as a function of radiation dose, with or without the 2 thiols, is shown in Fig. 7A, and indicate a PF[DSB] of between 2.1 and 2.2 at all levels of effect. The corresponding survival data (Fig. 7B) gave a PF[SF] of again between 2.1 and 2.2 at all levels of effect.

Clearly, there is a reasonably good agreement between the PFs determined in this way, suggesting that the calculation of PF values per se is probably not the cause of the disparity found in the earlier data (Table II). Furthermore, the PF method is a useful approach in that some idea of the magnitude of protection can be made; the lethal lesion plot, although greatly superior for determining the goodness of a correlation between two end-points, tells us nothing about the magnitude of the effect of a given drug.

b. The lack of correlation in the earlier studies may be due to either: (i) a true dose-dependency of DSB protection, perhaps reflecting a saturation of the ability of RSH to donate H-atoms or electrons or to scaverge OH° radicals at very high radiation doses, or (ii) an "apparent" dose dependency that may be related to an increasing contribution of SSBs or base damage (which are both poorly protected against) to that damage assayed as DSBs at the higher radiation doses. Each of these 2 effects are

extremely plausible, and in reality both of these processes probably contribute to the observed results.

In conclusion, it appears that when the truly appropriate measurements are made, there seems to be an excellent correlation between the effects of aminothiols on cell survival and DSB induction. At first, this sounds appealing, and any drug-screening program would have an obvious advantage in incorporating such an assay. However, in some respects, this result is mechanistically surprising since the implication is that DSB induction ALONE determines the survival level, and that factors such as cell recovery, cell progression, etc., which have been widely implicated as having an important role in determining cell survival, must have no effect in determining the subsequent protection. These factors will be discussed further in sections A5 and 6. Before going on to a discussion of the effects of the aminothiols on DNA repair processes, I will briefly outline a rather interesting aspect of thiol protection in general and then indicate some rather unexpected implications of this model.

The "fixation-repair" (F-R) model of radiosensitization and radioprotection--more specifically as it relates to oxygen and thiol compounds--was formulated many years ago by Alper and Howard-Flanders (15). Although many pieces of evidence are not fully explained by this model -- and indeed, many modifications of the F-R hypothesis are to this day being formulated -- the model in its most simple form has provided an extremely useful general model for discussing the O_2-effect. As shown in Fig. 8, the basis of this hypothesis is that peroxidative lesion fixation (F) reactions:

$$DNA^{\bullet} + O_2 \rightarrow DNAO^{\bullet}_2 \rightarrow DNAO_2H$$

occur in competition with chemical repair (R) reactions:

$$DNA^{\bullet} + RSH \rightarrow DNAH + RS^{\bullet}$$

Reaction of $DNA^{\bullet}$ with O_2 will enhance lethality, reaction with RSH will decrease lethality. But what will the outcome be in terms of DNA lesions? If we assume that (i) reaction with all lesions will occur with equal probability; and (ii) that DSBs correlate 1 to 1 with survival; then it is apparent that, for a DSB, a thiol will have two "opportunities" to scavenge a DSB (since repair of either potential strand break in a DSB will result in the disappearance of the DSB) whereas for an SSB there will only be one such "fixation-repair" opportunity. This will result in a distribution of products as shown in Fig. 8; not only will protection against SSB be much less efficient than for DSBs, but some percent of the potential DSBs will actually be converted to an SSB. Therefore, based on simple mechanistic considerations, the observation that:

$$PF[SF] - PF[DSB] >> PF[SSB]$$

appears to be reasonable, not forgetting that the PF[SSB] may be further reduced as a result of breakage of the DNA by RS• or RSO_2• radicals, as discussed earlier in section A2.

A4. Single-strand break repair

In our initial studies with WR-1065 (1) and WR-255591 (2) we performed detailed analyses on the effects of these 2 drugs on the repair of γ-ray-induced SSBs. We examined the effect of pretreatment only, post-treatment only, and pre- plus post-treatment, on SSB repair. Post-treatment with either drug appeared to inhibit SSB repair; however, this "inhibition" could be reversed by the addition of catalase (which removes H_2O_2) and desferal (which removes Fe), so this effect was probably a result of additional SSBs induced via OH-radicals generated from autoxidation of the drug as follows:

$$2RSH + O_2 \rightarrow RSSR + H_2O_2$$
$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\bullet} + Fe^{3+}$$

the latter reaction being the well-known Fenton reaction. Some further aspects of these reactions as they relate to SSB induction resulting from thiol autoxidation were discussed in our second Annual Report (16). Thus, post-irradiation incubation with aminothiols appears to have no "real" effect on SSB repair per se. This conclusion is supported by the fact that post-irradiation incubation with either drug had NO effect on the survival of the cells.

On the other hand, in cells that were treated with either WR-1065 or WR-255591 before irradiation, the subsequent rate of SSB rejoining was significantly slower regardless of the addition of agents to offset the effects of thiol oxidation. Such effects have been reported by other workers for both cysteamine (17) and WR-1065 (11) and have generally been interpreted as reflecting an inhibition by the aminothiol of a DNA repair process. However, various lines of evidence have lead us to conclude that this effect is perhaps not related to an "inhibition" of a DNA repair process: (i) There was no comparable effect on DSB repair (see section A.5) for either drug. (ii) There was no effect of a post-irradiation treatment with either WR-1065 or WR-255591 once the effects of thiol oxidation had been compensated for, as discussed above. This should not have been expected if the drugs inhibit, say, a repair enzyme, since in this protocol the thiol is actually present during the repair process.

Thus, we are left with the conclusion that the present--and perhaps also previously reported--effects of thiols on DNA repair may be artifactual in origin. The more significant effect of pretreatment on SSB repair is again probably not the result of an effect on DNA repair systems; rather, we believe that this effect could reflect the differential protection by WR-1065/WR-255591--or indeed, any thiol--against different types of SSB. In this case, the drug would have to protect differentially against the precursor radicals that lead to faster-repairing SSBs, so that

those SSBs remaining would contain a preponderance of slower-repairing types. A similar conclusion was reached by van der Schans et al. (18) for anoxic cells treated with cysteamine.

This conclusion is supported by many papers in the literature which show that thiols differentially donate H-atoms or electrons to various types of base or sugar radicals (e.g., 19,20). Furthermore, it should be remembered that the SSBs measured by alkaline elution at pH 12.1 will contain a significant contribution from alkali-labile base damage as well as from frank SSBs. Several reports have indicated that thiols such as cysteamine protect very poorly against base damage (7,18,21) compared to frank SSBs, so there may well be a greater contribution from these (slower repairing?) lesions in thiol-treated, irradiated cells. I will return to this concept later in section A.6 as it relates to PLD recovery.

A5. Double-strand break repair

In view of the widely proposed role for DSBs in determining cell survival, their repair by DNA-repair systems should be an important cellular event. Indeed, the level of DSB induction should only correlate with cell survival when there is no effect of the aminothiol on DSB repair. We measured DSB repair after a dose of 100 Gy (irradiation on ice followed by repair at 37°C) and found that neither WR-1065 (1) nor WR-255591 (2) had any measurable effect on the rate of DSB repair regardless of whether the drug was present before and/or after irradiation. In our studies of DSB induction at that time we were not finding a one-to-one correlation between DSB induction and cell survival, so we had anticipated that the drugs may enhance DSB repair. Of course, our subsequent finding that there was indeed a close to 1-to-1 correlation between cell survival and DSB induction at lower radiation doses (section A.3) made this observation of "no effect" on repair much more reasonable.

Incidentally, a recent paper by Radford (22) makes a very interesting point that care should be exercised when interpreting such DSB repair data since it appears that only a small fraction of the genome (~2% - the genes?) is a "target" for cell inactivation. Thus, measuring DSB repair in the whole genome, as we did here, would give no information on repair in the important DNA sequences.

In summary, we see no evidence to suggest that WR-1065 or WR-255591 exert any effect on DNA repair processes, other than perhaps indirectly by altering the distribution of lesion types on which the repair enzymes operate.

A6. Effect of thiols on PLD recovery.

We originally began to address the possible role of PLD recovery in radioprotection because: (i) of our observation that the PF[DSB] measured after high doses of γ-rays was always lower than the PF[SF] (Table II). Thus, the discrepancy between these two numbers could reflect enhanced cell recovery processes. (ii) of the reports by Grdina and Nagy (11) that WR-1065 reversibly

perturbed the progression of V-79 cells through their cell cycle. This, plus earlier reports of a similar effect with cysteamine (17), lended support to the suggestion of Brown (23) that inhibition of cell progression/DNA replication by aminothiols could allow additional time for the repair of potentially-lethal lesions.

This latter point is important, and greatly influenced the design of the experiments described below. Many workers had speculated that such an effect could be related to the binding of aminothiols to DNA, with a resulting stabilization of the DNA structure (23). Thus, the charge (Z) of a thiol should be an important variable in this respect. Aminothiols such as WR-1065, with Z = +2, should bind strongly to DNA and may thus be expected to exert a strong effect on PLDR (see reference 16 and literature cited therein). Thiols such as DTT, on the other hand, with Z = 0, should have little effect if this mechanism is appropriate.

Thiol	Z
WR-1065	+2
WR-255591	+2
WR-151326	+2
cysteamine	+1
DTT	0
GSH	-1

We attempted to answer this question through a series of relatively simple experiments. It is known that treatment of log-phase cultured cells with hypertonic (0.5 M) salt (HS) after irradiation "fixes" a sector of PLD in these cells and enhances radiation lethality (24). We had recently used this technique to assess the PLD repair capability of a series of X-ray-sensitive CHO-cell mutants (25). We reasoned that, if PLD was affected by aminothiols, then treating with HS should eliminate this effect and thereby differentially sensitize untreated and drug-treated, γ-irradiated cells. In fact, we anticipated that an HS treatment that eliminated the PLD component could reduce the PF[SF], perhaps even so that it would equal the PF[DSB] value. Our first experiments were done with WR-1065 (4 mM), as shown in Fig. 9. We were naturally intrigued when the HS treatment indeed appeared to sensitize both the control and WR-1065-treated cells but to a different extent. In fact, remarkably, the PF[SF] for salt-treated cells was ~1.6, i.e., very close to the PF[DSB]. Our original interpretation of these data (16) was that they may indeed represent a 2-stage protection process, namely a protection against DSB induction followed by a contribution from enhanced PLD recovery. We speculated that the enhanced PLD recovery could result from either: (a) effects on pH; (b) effects on cell progression; or (c) the effects of the drug on the spectrum of DNA lesions induced by γ-rays (16). This latter possibility was particularly intriguing in view of the known ability of thiol compounds to differentially repair (by H-atom or electron donation) different types of DNA radicals (19,20). Of

course, possibility (c) should be independent of the charge of a thiol and should apply equally to, say, WR-1065 (Z = +2) and DTT (Z = 0).

We did not immediately publish these data for several reasons. Most importantly, we still did not understand the mechanisms of PLD fixation by HS and we didn't know yet what the magnitude of the PF[DSB] would be at low doses of γ-rays (section A.3). The initial HS data with WR-1065 were intriguing enough that we continued with both lines of investigation for several months. As discussed in section A.3, it turned out that the modification of DSB induction did appear to correlate with cell killing at the lower doses, suggesting that the discrepancy between the PF[SF] and PF[DSB] values were <u>not</u> due to a PLD component but rather were due to a dose-dependence of the PF[DSB]. Nonetheless, we had consistently seen a differential sensitization of WR-1065-treated cells by HS, suggesting that the relationship between DSBs and survival could indeed be manipulated by PLD modifiers, although this now appeared to work in the <u>opposite</u> sense to our original expectation, i.e., HS <u>dissociated</u> the relationship between DSBs and survival in the <u>low-dose</u> range.

We therefore decided to examine some additional thiols to determine the generality of this effect. To assess the effect of thiol charge on this process we selected WR-1065 (Z = +2), cysteamine (Z = +1), and DTT (Z = 0). The results are summarized in Fig. 10 where the PF[SF] is plotted as a function of radiation dose for all 3 thiols, both without and with post-irradiation treatment with HS. All 3 drugs were affected to essentially the same degree by the HS treatment, there being an approx. 40% decrease in the PF[SF] as a result of the HS treatment at all radiation doses. Thus, we can conclude that this effect is independent of the charge on the thiol and is also a general phenomenon for a number of structurally unrelated thiols.

Our interest in PLD has also led us into an interesting area of research that has generated some revealing data. It is well established that the induction of chromosomal aberrations correlates well with both DSB induction and cell survival. Chromosome-level end-points had the attraction to us that they allow for repair and recovery processes to occur before damage is assessed, and may therefore be a good end point for assessing the effects of drugs such as the aminothiols which may affect these latter processes. The micronucleus (MN) assay is particularly convenient for measuring the formation of acentric chromosome fragments; however, it is difficult to apply this assay to agents which perturb cell progression, such as the aminothiols. It was therefore providential that Dr. William Brock in our department had been developing an adaptation of the MN assay which could circumvent this complication by using the agent cytochalasin, a drug which inhibits cell division but not nuclear division. Thus, it is possible to score MN induction selectively in binucleated cells that have only undergone one mitotic division.

Preliminary data were obtained with cysteamine and WR-255591; dose-response curves for MN induction in CHO cells with or without 6 mM WR-255591 or 10 mM cysteamine (Fig. 11) indicated that these drugs protected significantly against MN induction, although the PF[MN] for WR-255591 appeared to be somewhat less than that for survival (2.3) reported in our earlier paper (2). Based on our experience with the DSB induction data (section A.3), we noted that the MN data could only be measured at LOWER doses of radiation than the survival measurements had used. It was therefore possible either that (i) the PF[SF] would be lower at lower radiation doses, or (ii) the batch of WR-255591 used in the MN assay may have been less efficacious than the earlier batch used for the survival measurements. We therefore re-examined the protection of CHO cells by WR-255591 and cysteamine at lower x-ray doses, as shown in Fig. 12, and found that the PF was indeed lower than the old number. In fact, when we construct a "pseudo" lethal lesion plot analogous to that used with the DSB induction data (Fig. 13) there is a very good agreement between the lethal efficiency of MN with or without the drug, i.e., the drug modifies survival and MN induction in a similar proportion. These data therefore support the conclusions based on the low-dose DSB data (Fig. 6), and confirm that DSBs and MN are also modified in a similar ratio. I used the word "pseudo" above since in a true lethal lesion plot the DSB and survival data are performed on replicate sets of cells. With the MN assay the cells are treated with cytochalasin just prior to irradiation so survival cannot be determined on a replicate population.

As discussed above, effects on cell progression have frequently been implicated as a contributory effect to PLD recovery and radioprotection, although bearing in mind the close correlation between survival and DSB induction (section A3), this contribution may in fact be minimal. As an associated project we have used flow-cytometry to examine the effects of several thiols--WR-1065 (Z = +2), WR-255591 (Z = +2), cysteamine (Z = +1) and DTT (Z = 0)--on CHO-cell progression. Not all of these data have been analyzed and compiled as yet; however, it does appear that sometimes a small effect of the aminothiols could be detected, and sometimes their is no effect at all. Bearing in mind the low variance in measurements of cell survival, any effect for which the experimental variance of the technique itself is low (e.g., flow cytometry) should show a similar intraexperimental variation as does survival if the 2 effects are correlated. This does not appear to be the case for cell-cycle perturbation, suggesting that such perturbation may be cell-line dependent (11,26) and therefore not a general component of protection.

A7. Associated studies.

Several additional peripheral studies were performed during the course of this contract. These studies--notably the effect of GSH and polyamine depletion on protection by thiols and an

examination of DNA damage resulting from thiol oxidation products--were discussed at length in our Annual Report #2 (16), and little further work was performed since that time. The polyamine and glutathione data were presented by my colleague Ariela Prager at the Radiation Research meeting in April 1988. Our data showing the effect of WR-255591 on 42 MeV fast neutron-induced cell killing and SSB DSB induction discussed at length in the Annual report #2 (16) have now been written up and accepted for publication (27).

A8. Summary and conclusions from in vitro data.

The real purpose of these in vitro studies was to establish the relationships between the modification of proliferative cell death and DNA damage and repair processes in a relatively well-defined system where we could control all variables, ultimately with a view to using these data to elucidate the importance of these various processes in radioprotection in vivo. DNA SSB induction has long been known to correlate poorly with cell killing. We were nonetheless surprised by the extent to which these various thiols failed to modify SSB induction since our original expectation was that, even if SSBs were not lethal, all of the major processes that are believed to contribute to protection of the DNA--$OH^{\bullet}$-radical scavenging, repair of $DNA^{\bullet}$ radicals by H-atom or electron donation, or indeed O_2-depletion--should all protect the DNA from SSB induction to an extent that may even be expected to be greater than the PF[SF] value. Clearly this was not so, and a wide variety of thiols gave a similar low PF[SSB]. We believe that this can probably be explained in terms of artifactual DNA breakage from radiolytic products in irradiated, thiol-treated cells, from base damage (which has an intrinsically low PF) contributing to the measurement of SSBs, and from a simple radiobiological consideration of the fixation repair model (Fig. 8), as discussed in section A2.

With respect to SSB repair (section A4) the above caveats really cause us to question the degree of inference that should be drawn from such measurements. The post-irradiation effects are massively complicated by the possibility of post-irradiation DNA-damaging events, while the measurement of repair after pretreatment may reflect the fact that thiols are differentially protecting against various types of base or sugar damage. This perhaps leads to a simpler conceptualization of the "heterogeneous" nature of the PF[SSB] which represents an average for various types of damage (H-atom abstracted sugar radicals and bases) that may be extremely well protected against, and other types of radical (e.g., resonant guanyl radicals or forms of alkali-labile base damage not giving rise to frank SSBs) that are only poorly protected against.

We appear to have turned full circle in our consideration of the DSB induction data as better methods have been developed for their detection. The method described by Radford (7,8) is extremely powerful in many ways, and a comparison of the high

versus low-dose DSB induction data have lead to a much better understanding of the measurement and significance of these lesions. Clearly, the measurement of DSBs in stem cells in vivo is a major direction for future consideration. Indeed, we were beginning to address this question at the termination of this contract and will be glad to keep the appropriate office of the U.S. Army notified of any developments if this should be of use in their future research programs.

We are left with an intriguing paradox. The modification of cell survival and both DSB and MN induction appear to correlate fairly well with each other. However, postirradiation treatment with HS dissociates these 2 end-points, suggesting that the magnitude of PLD recovery is different in control and thiol-treated cells. Indeed, there is much circumstantial evidence to suggest that this should be so! Of course, if DSB induction correlates with survival, then there should be no differential effect on repair or recovery processes!

B. IN VIVO STUDIES

A major goal of this research has been to examine the extent to which the relationships determined in in vitro cell culture systems can be extrapolated to describe the behavior of tissues irradiated in vivo when animals are treated with various radioprotective aminothiols or their phosphate-blocked derivatives. The first objective here was to establish suitable assay systems for the various tissues of interest--jejunum, bone marrow, and brain. Our earliest studies in this area had resulted in the development of a variation of the alkaline elution assay which enabled us to examine DNA SSB induction and repair in these tissues (4). However, this assay, which used the fluorescent dye Hoechst 33258 to assay the eluted DNA (28), characterized the collective response of all of the cells within the tissue. We reasoned that an assay that was going to be of any value in describing the biological response of a tissue would have to tell us something about the response of that small subpopulation of radiosensitive target cells in the tissue. As a first approach to this goal we devised an assay where the animals were labeled with ^{3}H-TdR 6 hr prior to irradiation so that we could selectively examine SSB induction and repair in the proliferating cells of the mouse bone marrow and jejunum; this assay was rigorously characterized and the results published in a detailed manuscript (5).

B1. Studies of the effects of WR-1065 and WR-2721 on the radiosensitivity of the mouse jejunum.

Our next study involved the use of this assay to characterize the effects of WR-2721 and its free thiol, WR-1065, on SSB induction and repair in the mouse jejunum. Again, these studies were reported in an in-depth publication (29). To briefly summarize these studies, we found that both WR-1065 and WR-2721 gave marked protection to this tissue in a biological assay for stem-cell survival, viz, the crypt microcolony assay devised by Withers and Elkind (30). Rather surprisingly, under these same conditions we found that neither WR-2721 nor WR-1065 gave much protection against SSB induction.

We therefore examined the effect of these 2 drugs on SSB rejoining and, again rather surprisingly, found that both drugs markedly inhibited the rejoining of SSBs by the proliferating cells of the jejunal crypts. There are several possible explanations for this observation:

(a) WR-1065/2721 could directly inhibit those enzymatic pathways responsible for the repair of SSBs.

(b) the effect could be analogous to that observed for WR-1065 (1) and WR-255591 (2) in cultured CHO cells that were pre-treated with the aminothiol and which we attributed to a change in the proportions of different types of lesions induced in the presence or absence of the drug. However, since there was no change in the level of SSB induction in vivo, this is unlikely.

(c) it could be analogous to the slower SSB-repair observed in CHO cells if catalase and desferal were not included in the protocol, i.e., due to thiol oxidation products.
(d) $RS^{\bullet}$ and $RSO_2^{\bullet}$ radicals could be generated in irradiated thiol-treated tissues, as discussed for CHO cells in section A2. These radicals could then produce additional DNA SSBs.
(e) WR-2721 is known to produce a hypothermic effect, and the decrease in body temperature could result in a decreased rate of SSB repair.

The answer is probably a combination of factors c-e, and is unlikely to represent a true inhibition of the SSB repair processes. These data--and particularly comments b and c--do illustrate some of the difficulties of working with in vivo systems, and emphasize one of the original provisions of the studies that were performed under this contract, viz., the importance of the mechanistic studies in cell culture, where conditions can be carefully controlled (in this case temperature, H_2O_2/Fe levels, etc.), in ultimately interpreting the more artefact-prone in vivo data.

B2. WR-3689 as a protector of the mouse jejunum.

It was apparent from this study with WR-2721 in the mouse jejunum that the relationships in cultured cells could not be readily extrapolated to the in vivo situation. We therefore examined a second drug, WR-3689, the N-methylated derivative of WR-2721, whose free-thiol is the compound WR-255591 described in our in vitro studies (2). The results of these studies were written up and submitted to the British Journal of Cancer (31); this manuscript was revieved and revised, and is awaiting final decision. The material in this study (31) was expanded somewhat relative to the earlier WR-2721 paper (29). The reason for this was basically that we felt that, if the relationships that we determined from cell culture were going to be directly extrapolable to tissues, then the same biological end-point--the loss of proliferative capacity of the cells--must be used in both situations. The real biological end-point of relevance in these studies--lethality or loss of tissue function--must therefore be shown to be related to the loss of proliferative capacity of the stem-cell population. While there is a reasonable amount of evidence in the literature suggesting that functional assays (LD50/7) and clonogenic assays (crypt microcolony assay) for jejunal stem cell survival correlate well for radiation alone (e.g., 32) and for WR-2721 (e.g., 33), the agreement for WR-2721 is far from perfect, and Hanson (33) showed that for prostaglandin E_2, a non-thiol radioprotector, the relationship could be completely dissociated. We therefore felt that it was important to determine how closely the modification of gut LD50 and crypt-cell survival by WR-3689 was correlated. The results (Fig. 14) suggest that, at least when LD50 at 10 or 11 days after whole-abdomen irradiation was compared with the radiation dose required to reduce crypt-cell survival to 50-5 crypts per circumference, there was a good agreement, although other

end-points, such as the D_0 of the survival curves, did not correlate so well.

When we used the alkaline elution assay to examine the influence of WR-3689 on SSB induction in the mouse jejunum we found essentially no effect, as shown in Fig. 14, although the now general impairment of SSB rejoining was observed (16,31). The possible reasons for this effect will be discussed in section B4, but first I will discuss our results with mouse bone marrow, since these conclusions from studies with different tissues are intimately related.

B3. WR-2721 as a protector of bone marrow.

A preliminary account of these data was presented at the recent 4th International Congress on Radio-Oncology in Vienna (September-October, 1988) and published in the proceedings of that symposium (34). Briefly, we wanted to examine bone marrow because we felt that a key to understanding the effects of these drugs *in vivo* may be the level of tissue oxygenation. Based on our previous experiences (35) we anticipated that bone marrow stem cells may behave as though they were better oxygenated than those of the jejunum. The results that we obtained (Fig. 15) were intriguing insofar as there was now clearly a significant protection of this tissue by up to 400 mg/kg i.p. of WR-2721 with respect to SSB induction, although as shown in Table III the correlation between the effect on SSB induction and clonogenic (spleen-colony) or functional (LD50/30) assays for bone-marrow stem-cell survival was not of a one-to-one nature. The stem-cell survival data were obtained by my colleague Dr. Elizabeth Travis with whom I have collaborated on many aspects of this program (36).

B4. Summary and conclusions from the in vivo studies.

First, I would like to consider the *in vivo* PF[SSB] data in relation to the *in vitro* data, as shown in Table III. The low PF[SSB] values were not anticipated at the outset of these studies, and at first we thought that there must be some very odd radiobiological differences between cultured cells and tissues--for example, differences in oxygen levels coupled with the fact that the k-curves for SSB induction and cell survival may not be coincident, as discussed elsewhere (16,29). The possibility was that we may be dealing with intermediate levels of oxygenation in tissues where the relative position of the k-curves may be such that altering the RSH concentration could markedly change cell survival and yet have little or no effect on SSB induction (29). This still didn't seem very likely. However, once we had accumulated data with CHO cells for 5 different thiols (section A2)--WR 1065, WR-255591, WR-151326, cysteamine and DTT--we found that the same situation, i.e., PF[SSB]<<PF[SF], existed under these conditions too. Thus, this situation appears to be a predictable one rather than being an artifact or tissue-specific phenomenon, and possible reasons were discussed in sections A2 and B1.

C. Specific recommendations.

I believe that the studies described in this report are giving a very strong message with respect to the design of future studies. What is clearly needed are assays for DSB induction or chromosomal aberrations in vivo. The development of these assays should bear 2 important criteria in mind:

a. Sensitivity: The studies with CHO cells outlined in sections A3 and A6 strongly indicate the importance of measuring DSBs or chromosome aberrations over the same radiation dose range over which the biological end-points are being measured. These measurements should ideally be made on the same population of cells. There is no justification for extrapolating PF values among studies which use significantly different ranges of radiation doses.

b. Specificity: These assays must ultimately be capable of identifying DNA damage and survival in the relevant cell populations of the tissue. In the case of bone marrow and jejunum, this objective would be simplified because of the generally accepted identity of the radiosensitive target cells as the stem-cell population. For late-responding tissues this issue is much less straightforward, with there being a lack of a defined stem-cell population and the possibility of both parenchymal and stromal/vascular components. Bone marrow probably represents the most suitable tissue to begin such studies for several reasons:

(i) it is amenable to study by filter elution methods which will be particularly useful if the neutral elution methodology--which is currently the most sensitive assay for DSBs--can be extended to tissues.

(ii) it is easily dissociable into a single-cell suspension, an important prerequisite for future studies using flow cytometric methods.

(iii) several convenient assays for the survival of different stem-cell populations in vivo are available (e.g., the various spleen colony assays).

(iv) the technology in terms of using cell-type specific surface markers and fluorescent monoclonal antibodies to these antigens is already at an advanced stage of development and is already being used with flow-cytometric methods. Similar technologies are becoming available for the CNS through the efforts of our collaborator, Dr. Albert van der Kogel, in the Netherlands.

Such new technologies must surely be the key to really understanding the effects of radiomodifying agents on tissues at the level of relevant target populations. Of the various parameters identified to date, intracellular oxygen concentration appears to be a major factor in radioprotection and in determining the relationships between DNA damage and cell killing. Such studies will be best approached through the initial use of in vitro systems where the drug and oxygen concentrations can be carefully regulated, as outlined in my

Annual Report #2 (16). This information may ultimately be useful for understanding the behavior of animal tissues irradiated *in situ*.

Finally, some attempt should be made to determine the intracellular forms of the drugs that are responsible for these various effects. All of our previous studies have involved endpoints such as cell survival or DNA lesions, although exactly what form(s) of the drugs are responsible for these effects has not yet been determined. HPLC would be the methodology of choice to identify intracellular forms of the various drugs.

D. EXPERIMENTAL PROCEDURES

For a detailed description of the methodologies used in this contract, please see our previous Annual Reports (16,36).

D1. Cell Culture Methods

CHO cells are maintained and treated in exponentially growing monolayer culture at 37°C in a humidified 5% CO_2 - 95% air atmosphere in McCoy's 5A medium (Hsu's modification) supplemented with 15% fetal bovine serum. For the DNA damage studies cells are labeled for 24-36 h with 2-^{14}C-TdR (0.01 μCi/ml; 50 mCi/mmol) followed by a 6-h incubation with label-free medium to chase the label into high molecular weight DNA.

D2. Cell Survival

Cell survival is determined using a clonogenic assay, surviving cells being assayed by their ability to produce colonies of 50 cells or more.

D3. Mice

C_3H mice are maintained in a specific-pathogen-free breeding colony. Mice of between 12 and 16 weeks of age and weighing approximately 30 g are used for all studies. For the DNA SSB measurments, mice are injected i.p. with ^{3}H-TdR 6 h prior to irradiation. The radioprotective agent is administered i.p. at various times relative to the irradiation. Animals are sacrificed by cervical dislocation at various times after irradiation, and the tissues are removed and immersed in ice-cold PBS containing 5 mM EDTA to inhibit any SSB repair. Suspensions of jejunal epithelial cells and of femoral bone marrow cells are obtained as described previously (16).

D4. Alkaline Elution In Vitro

SSBs in CHO cells are measured using alkaline elution (3). 8×10^5 ^{14}C-labeled cells are layered onto 25-mm diameter, 2-um polycarbonate (PC) filters and lysed with 10 ml of SDS lysis solution containing proteinase K. The DNA is subsequently eluted in the dark with tetrapropylammonium hydroxide (TPAH) containing 0.02 M EDTA (free acid), pH 12.1, at a flow rate of 0.04 ml/min. Fractions are collected every 90 min for 15 h. Any DNA retained on the filter or remaining in the filter holder or barrel at the end of the elution time is recovered as described (16). The amount of DNA in each sample is assayed by liquid scintillation counting.

D5. Neutral elution

In the initial phases of this contract DNA DSBs were measured using the conventional neutral elution method which uses a pH of 9.6 (10) or with a modification of that technique in which a pH of 7.0 is used for the eluting buffer and for all other solutions (1). This assay required the use of supralethal doses of radiation (25-100 Gy). In the late phases of the project the high-sensitivity assay described by Radford (7,8) was used exclusively. In this assay, ^{14}C-labeled cells were irradiated (control cells with up to 10 Gy; drug-treated cells

with up to 30 Gy) and then split into two fractions. Fraction 1 was plated for cell survival; fraction 2 was analyzed by neutral elution for DSB levels, triplicate samples being run for each sample.

D6. Alkaline elution in vivo

The alkaline elution technique has been adapted for measuring SSBs in both the total and proliferating cell populations of the mouse jejunal epithelium and bone marrow after irradiation of the tissues in vivo (5). 5×10^6 cells are layered onto a 25-mm diameter, 0.8-um pore PC filter and lysed with a solution containing either Sarkosyl or SDS and proteinase-K. The DNA is eluted in the dark with 0.1 M TPAH containing 0.02 M H EDTA, pH 12.1, at a flow rate of 0.5 ml min. Any DNA retained on the filter at the end of the elution time is recovered (16) and the DNA concentration in each sample is assayed either fluorometrically using Hoechst 33258 (35) or by liquid scintillation counting. The data obtained using the radioactivity assay characterize DNA-damage in the proliferating cells while the data obtained using the fluorometric assay measure SSBs in all of the cells in that sample of tissue.

D7. Calculation of strand-scission factors

The relative number of strand breaks is determined by the equation: $SSF = -\log(f_x/f_o)$, where f_o and f_x are, respectively, the proportion of DNA retained on the filter for the unirradiated control and for the irradiated sample. For SSBs, an eluted volume of 21 ml is used for the calculation. For the high-dose DSB assay a larger volume of 31.5 ml is used in order to reduce the effect of analyzing profiles in the transition region between first-order elution and the subsequent slower elution. However, in the later low-dose DSB assay we reverted to using a volume of 21 ml.

D8. Radioprotective drugs and radiation treatments

For the in vitro studies, drugs are dissolved in growth medium immediately prior to use and sterilized by filtration. For the in vivo studies, the drugs are dissolved in physiological saline immediately prior to injection i.p. All irradiations are performed using a ^{137}Cs-irradiator with a dose rate of 5 Gy/min.

D9. Gut microcolony assay

The principles of this assay have been discussed in detail (30). Jejunal cross-sections are prepared for histological examination 3.5 days after irradiation. The number of regenerating crypts are estimated by microscope, and converted to the number of surviving cells by applying a Poisson distribution correction function.

D10. Spleen-colony assay

The spleen-colony assay (37) was used to examine the effects of radiation on the survival of bone-marrow stem cells. Mice were irradiated with graded doses of radiation and 8 days later

their spleens were removed and fixed in Bouin's solution. The number of gross surface spleen colonies were then counted.

D11. Animal survival assays

The assays for animal survival after doses that lead to death from either gastrointestinal damage (LD50/7 after whole-abdomen irradiation) or bone marrow depletion (LD50/30 after whole-body irradiation) were described in detail elsewhere (31).

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P: FIGURES AND TABLES

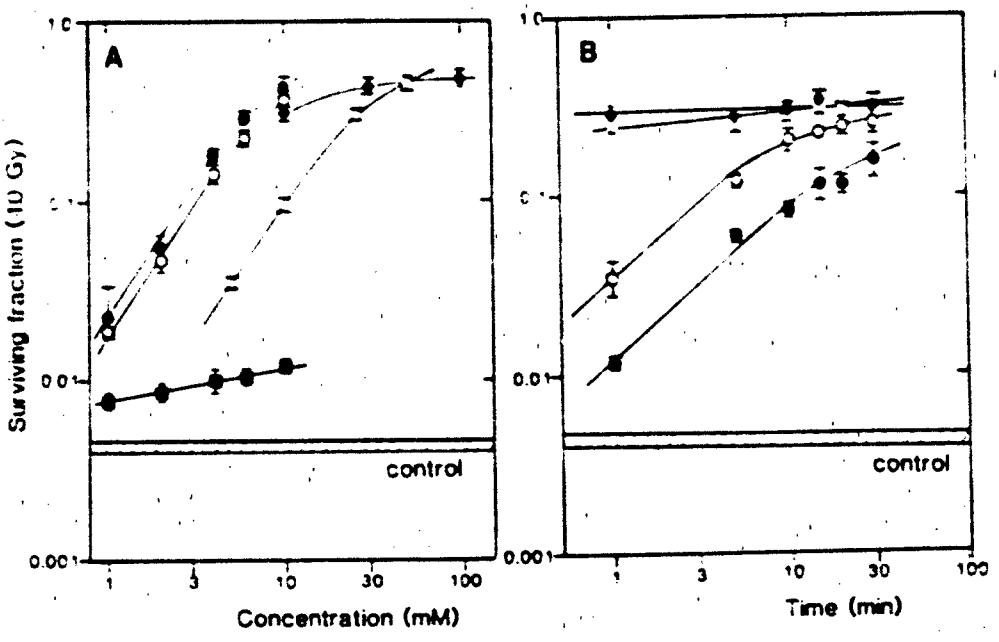


FIGURE 1: (A) Effect of varying concentrations of either WR-1065 (●), WR-255591 (○), WR-3689 (■), DTT (◇) or cysteamine (◆) on the survival of CHO cells irradiated with 10 Gy of γ-rays.

(B) Effect of varying lengths of exposure to these same thiols on radioprotection after 10 Gy of γ-rays.

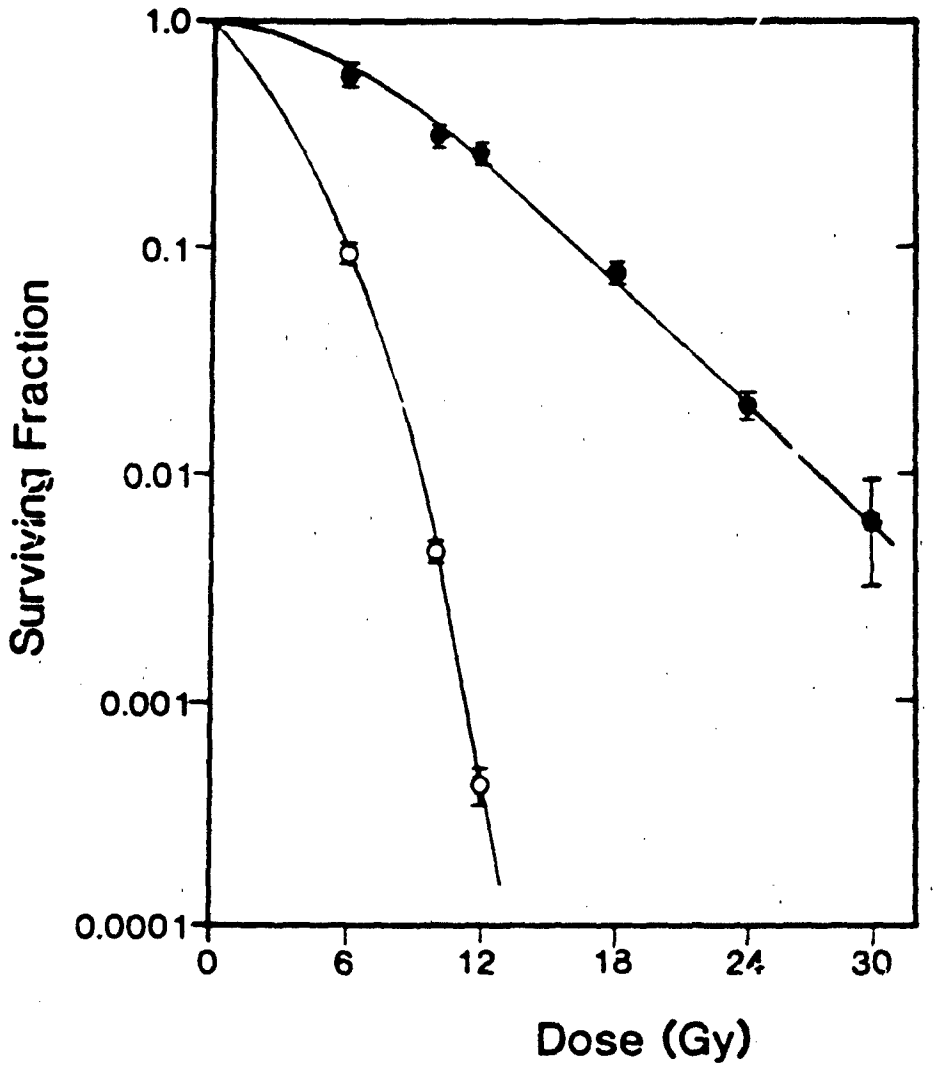


FIGURE 2: Effect of a 30-min pretreatment with 10 mM cysteamine on the γ-ray survival curve for CHO cells irradiated at 37°C: control cells (○); cysteamine-treated cells (●).

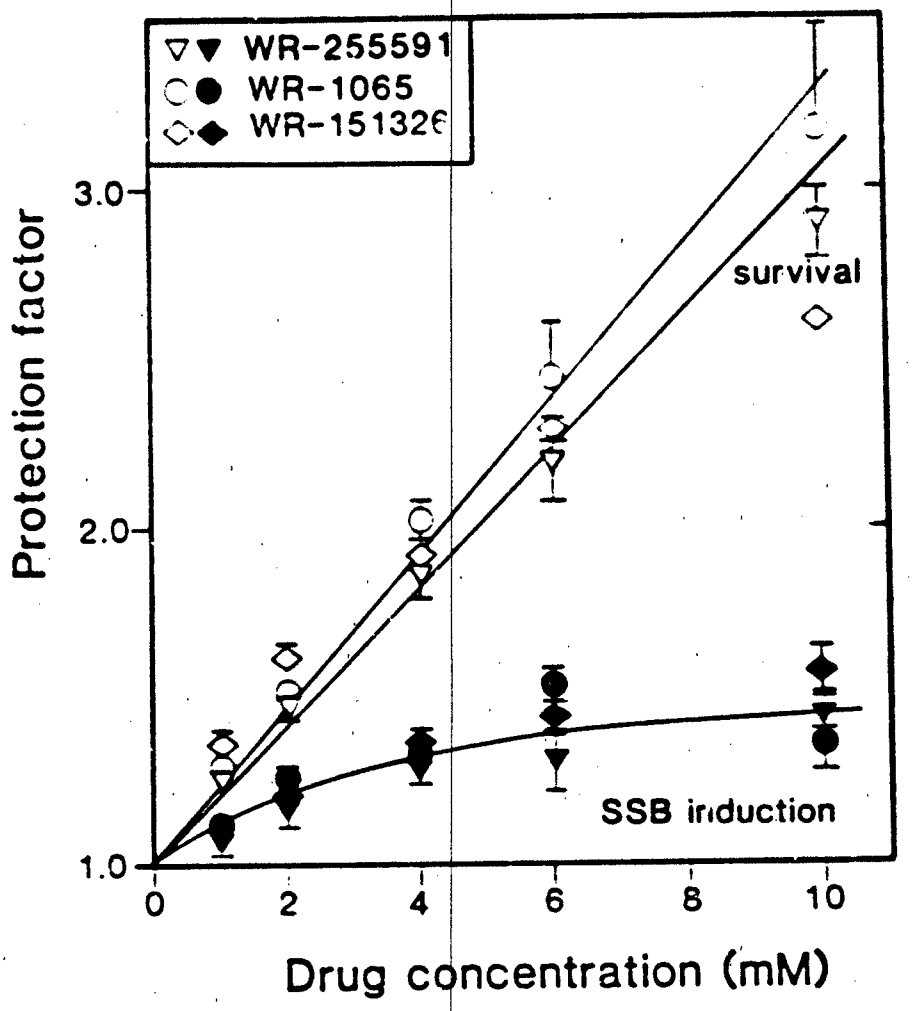


FIGURE 3: Effect of WR-1065 (○, ●), WR-255591 (▽, ▼), and WR-151326 (◇, ◆) on the yield of γ-ray-induced DNA single-strand breaks (solid symbols) and on the survival (open symbols) of CHO cells irradiated at 37°C.

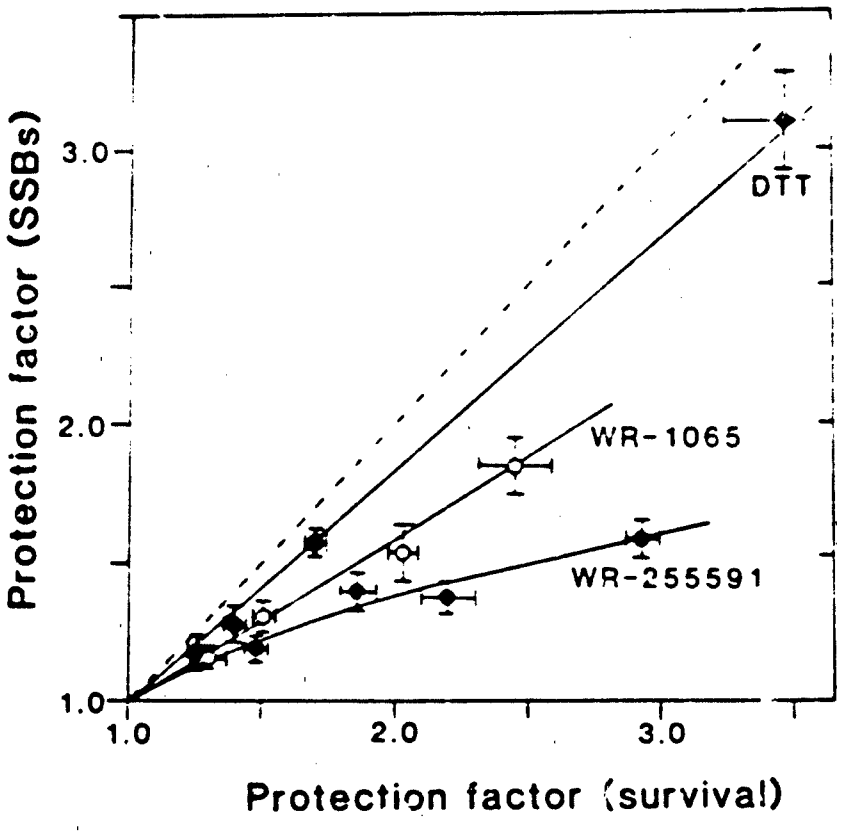


FIGURE 4: Correlation curves showing the relationship between the protection factor for DNA single-strand breaks and cell survival for CHO cells pretreated with DTT (◆), WR-1065 (_), or WR-255591 (●).

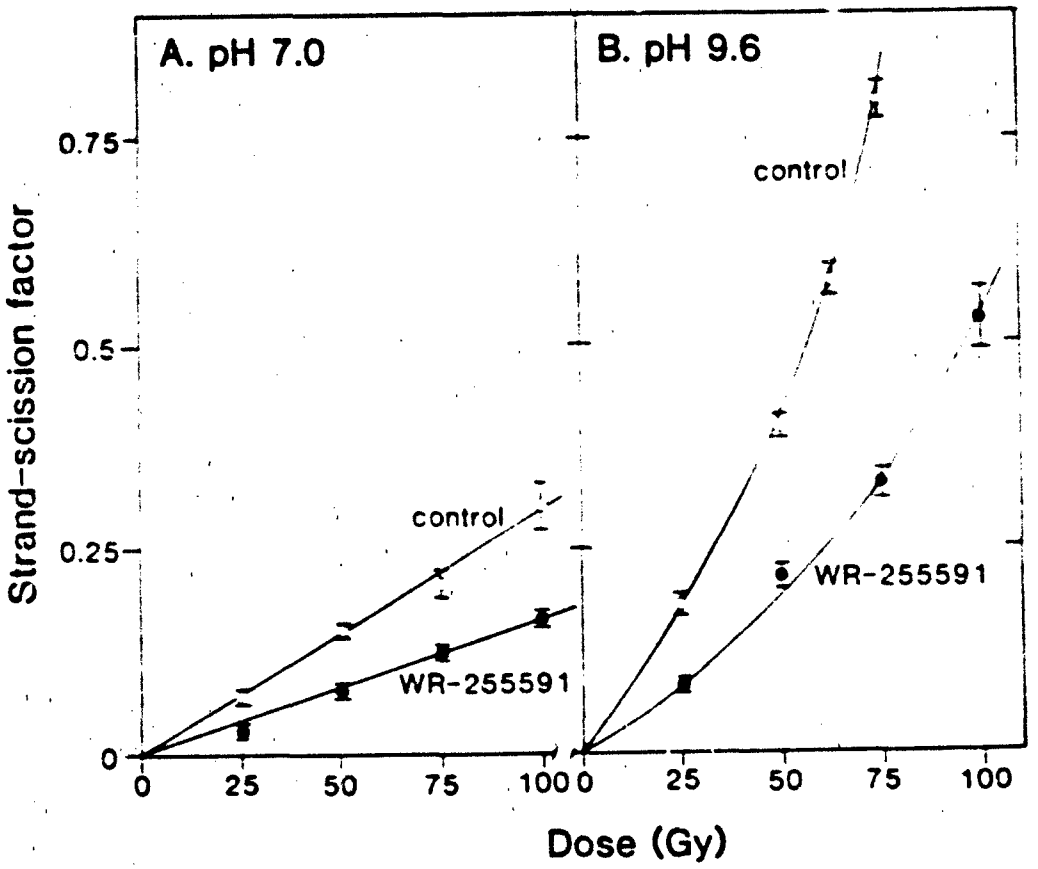


FIGURE 5: Effect of 6 mM WR-255591 on the yield of γ-ray-induced DNA double-strand breaks in CHO cells assayed at either (A) pH 7.0, or (B) pH 9.6.

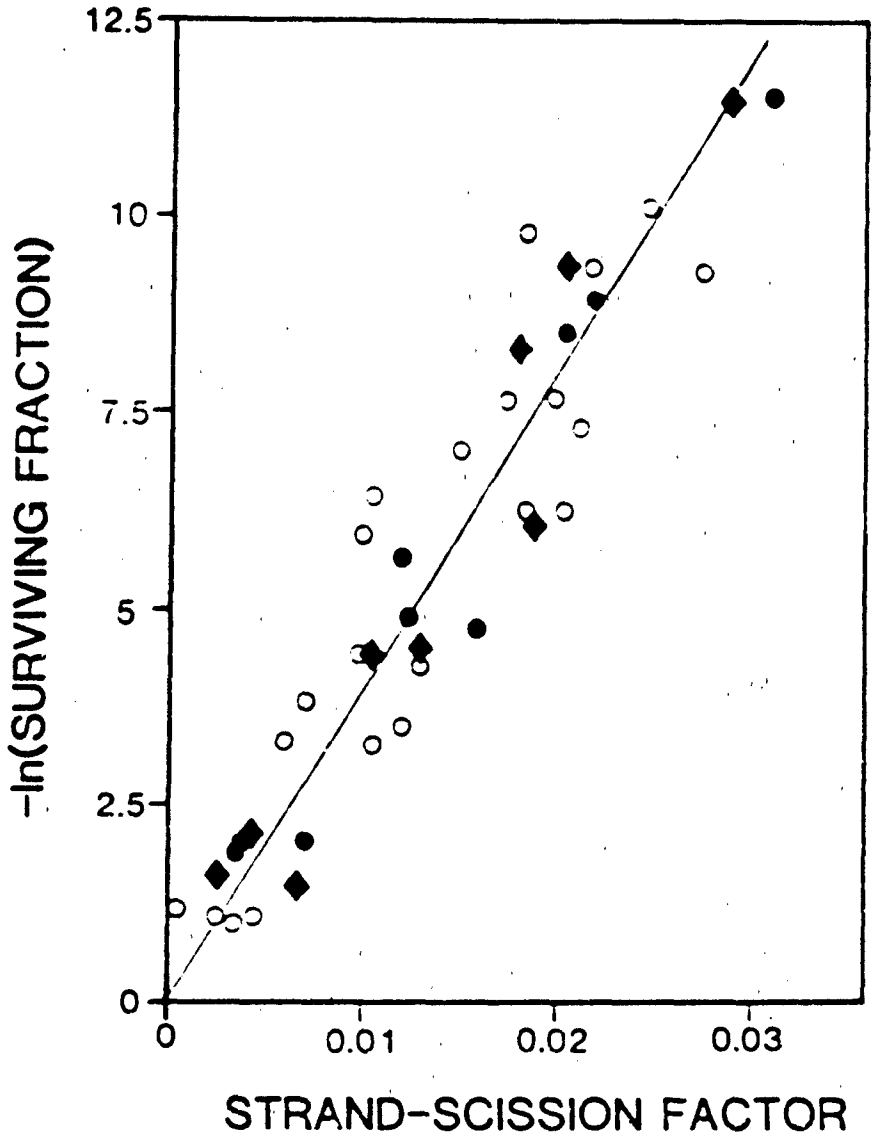
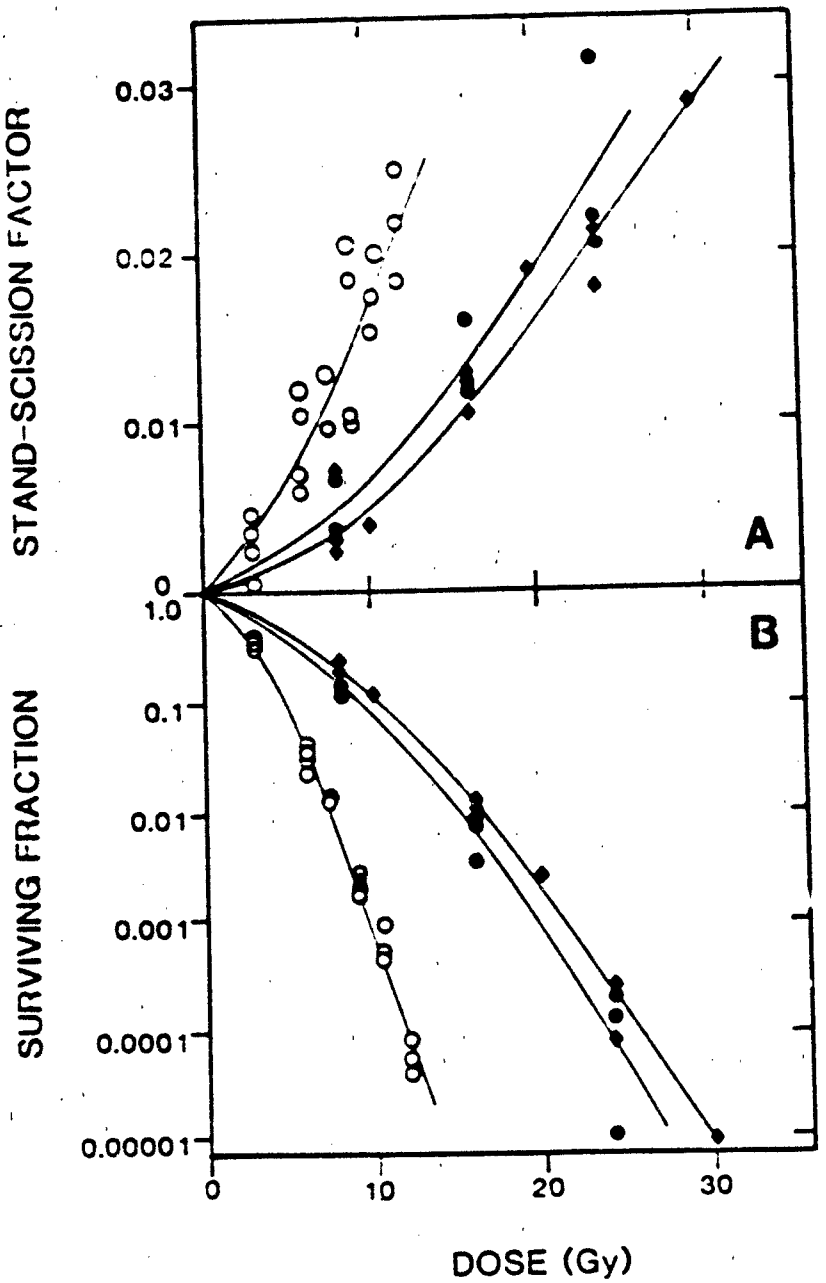
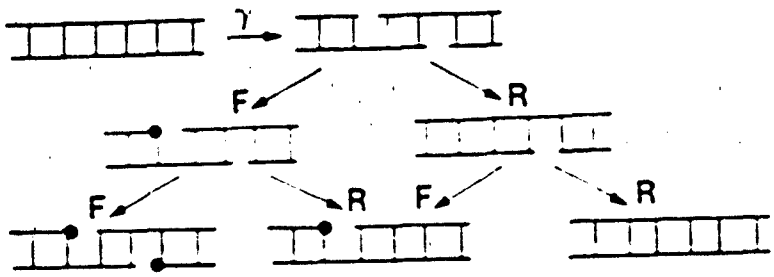


FIGURE 6: Lethal lesion plots for untreated CHO cells (○) or for cells treated with WR-255591 (◆) or WR-1065 (●) relating -ln(surviving fraction) to the level of DSB induction (measured at pH 9.6).

FIGURE 7: Effect of 6 mM WR-255591 (◆) and 4 mM WR-1065 (●) on (A) the yield of γ-ray-induced DNA double-strand breaks at low doses, and (B) on cell survival. In both cases the open circles (○) represent control cells (no drug).



a. Double-strand break



b. Single-strand break

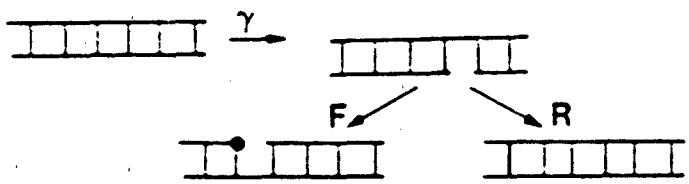


FIGURE 8: Idealized application of the fixation (F)-repair (R) model to protection against single- and double-stranded DNA break precursors by thiols. F-processes result from reaction with O_2, the fixed damage being represented by the solid circle ( ). R-processes result from reaction with the thiol.

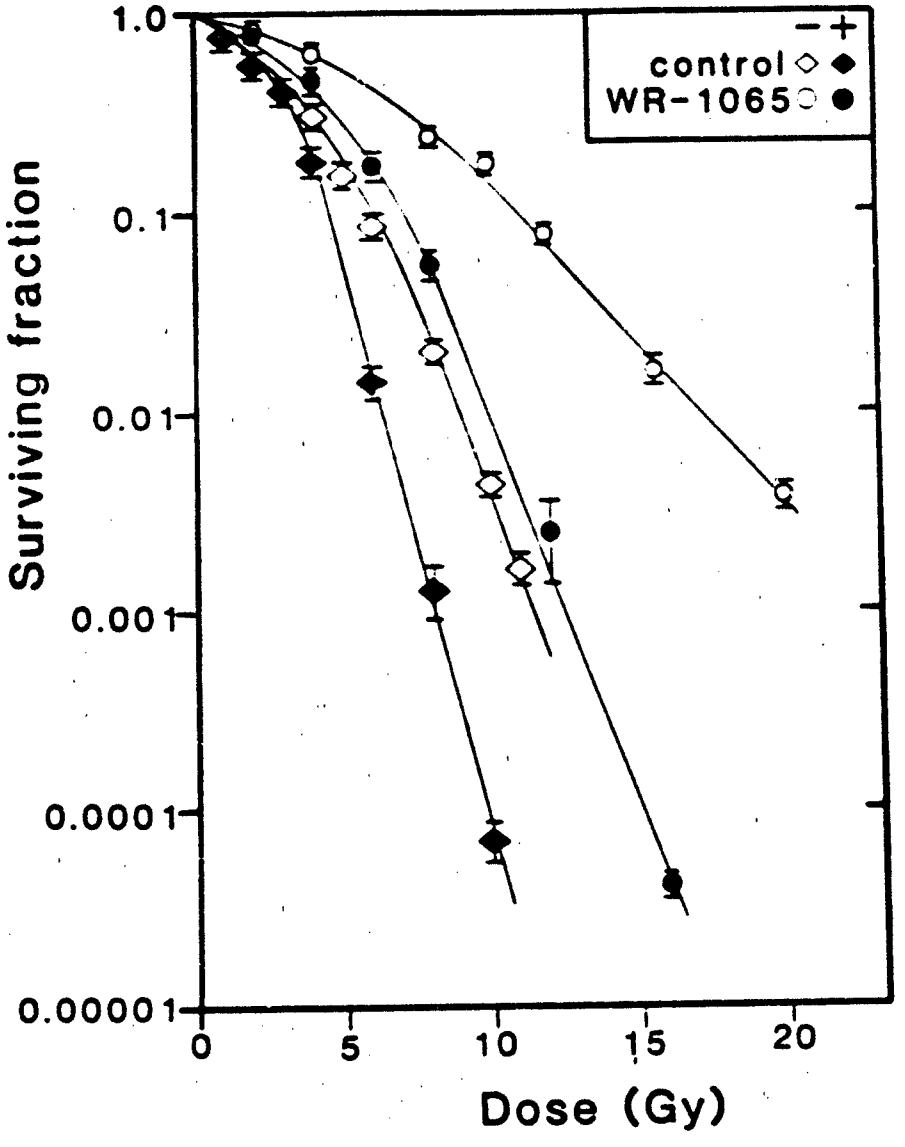


FIGURE 9: Effect of immediate post-irradiation treatment with hypertonic salt on the survival of log-phase CHO cells. Open sumbols are for normal plating conditions. Solid symbols are for cells treated with 0.5 M salt for 20 min immediately after irradiation.

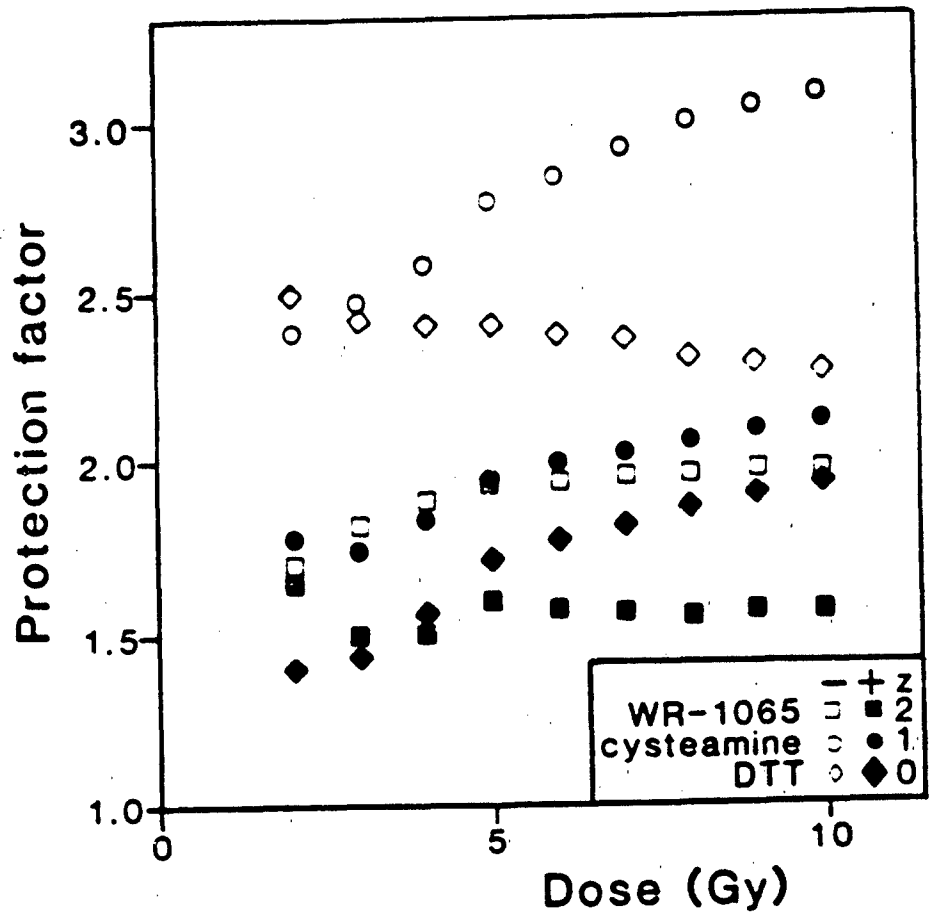


FIGURE 10: Protection factors for CHO cells treated with either DTT (◇◆), cysteamine (○●), or WR-1065 (□■), as a function of radiation dose. PFs are shown for cells either with (solid symbols) or without (open symbols) immediate post-irradiation treatment with hypertonic salt.

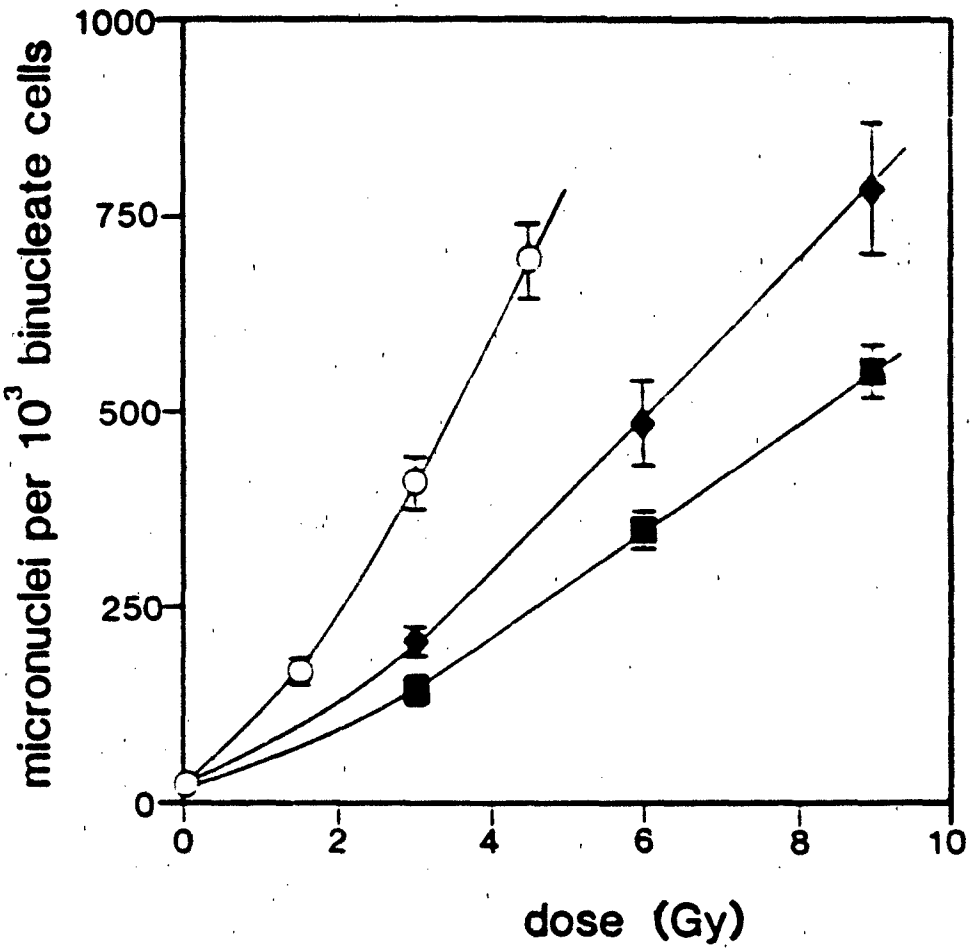


FIGURE 11: Dose response curves for micronucleus induction in CHO cells (○) or following a 30-min pretreatment with either 6 mM WR-255591 (◆) or 10 mM cysteamine (■) prior to γ-irradiation.

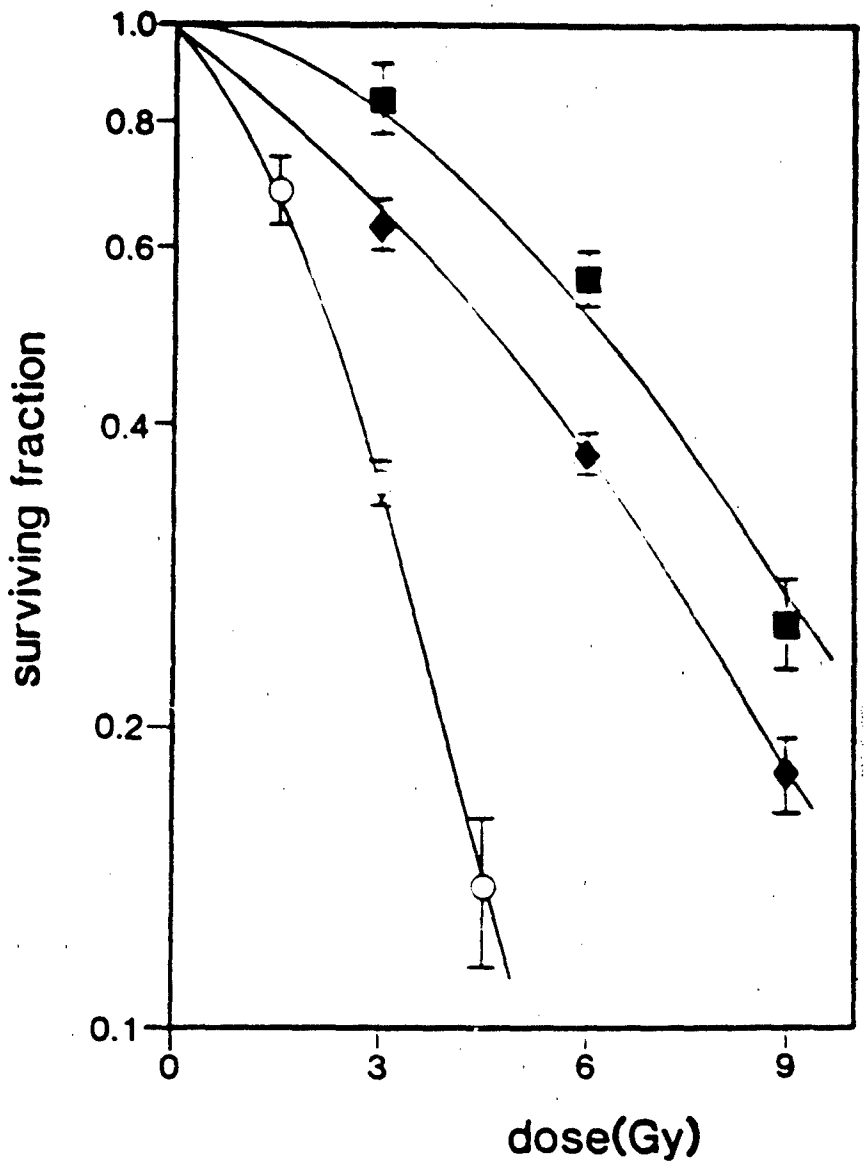


FIGURE 12: Protection against killing of CHO cells by 6 mM WR-255591 (◆) or 10 mM cysteamine (■) at low doses of γ-rays.

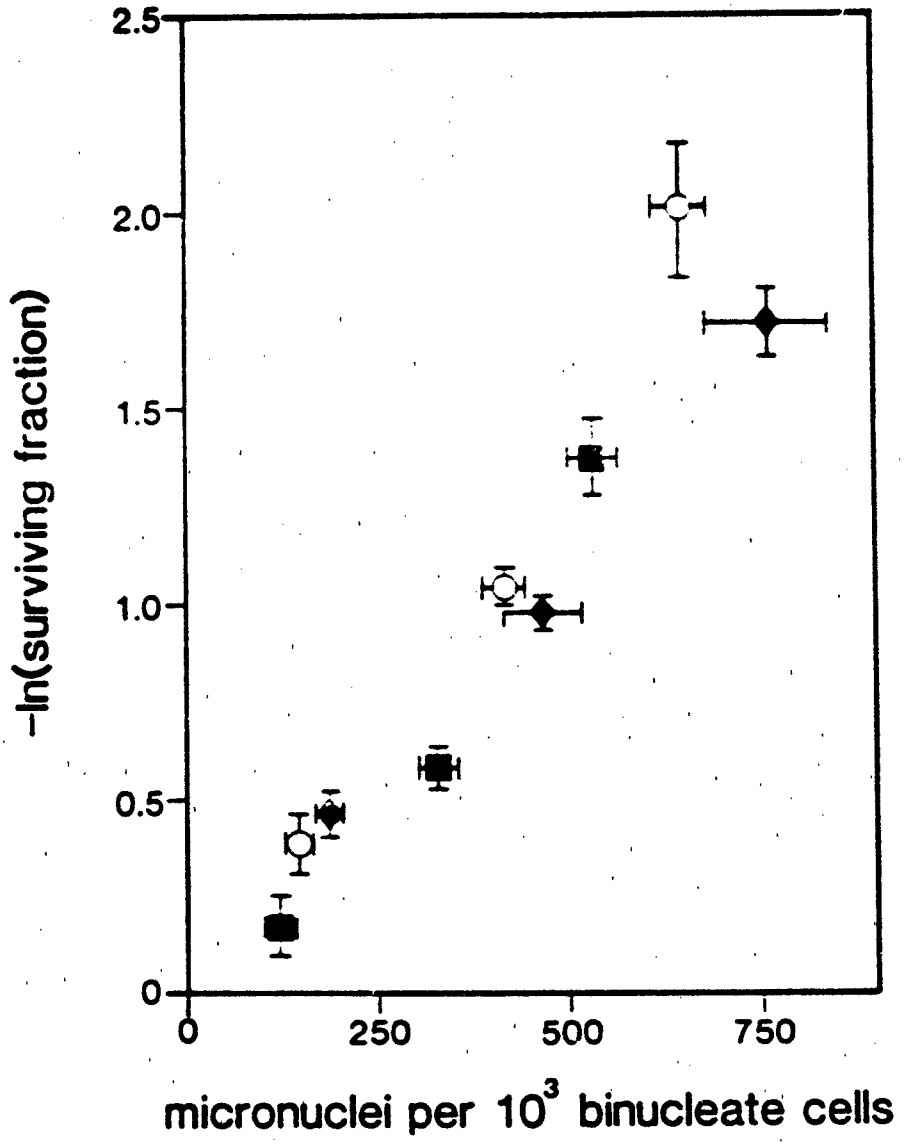


FIGURE 13: "Pseudo" lethal lesion plot relating -ln(surviving fraction) to the level of micronucleus induction. Treatment conditions were as follows: (○) controls; (◆) 6 mM WR-255591; and (■) 10 mM cysteamine.

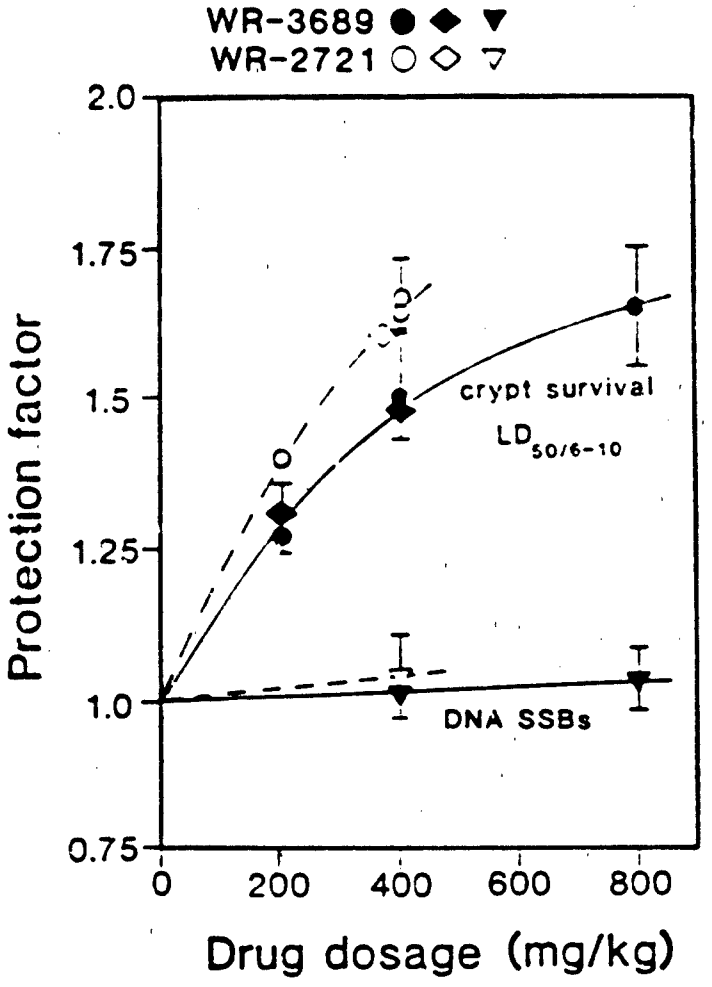


FIGURE 14: Effect of WR-3689 and WR-2721 on the survival of crypt cells (circles) or animals (diamonds) and on the yield of DNA SSBs (triangles) in mouse jejunal cells.

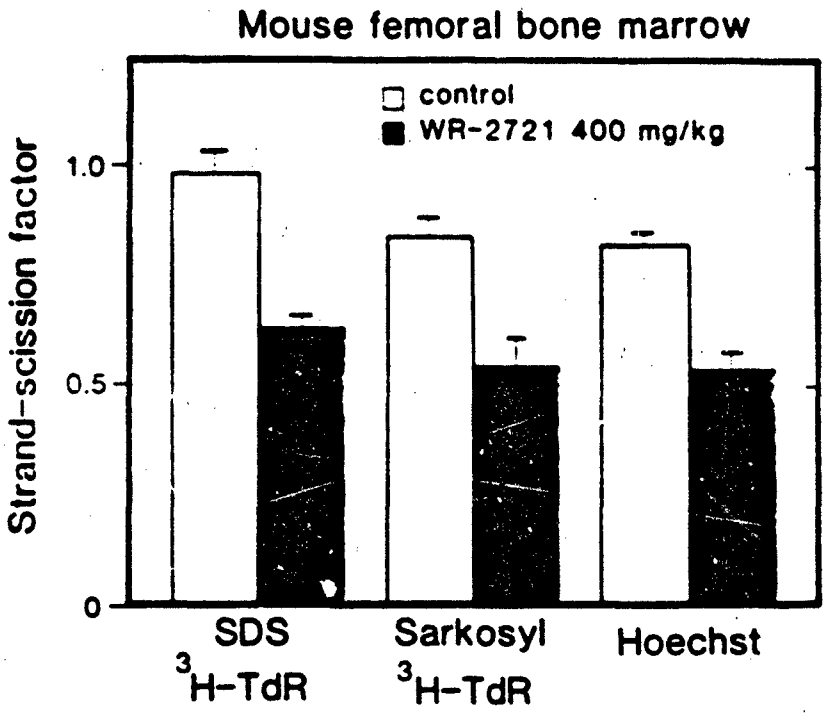


FIGURE 15: Effect of WR-2721 on the initial yield of γ-ray-induced DNA single-strand breaks in whole mouse bone marrow and in the proliferating bone marrow cells.

Table I: Structures of the Compounds Used in this Study.

WR-255591	$CH_3NH(CH_2)_3NHCH_2CH_2SH$
WR-2689	$CH_3NH(CH_2)_3NHCH_2CH_2SPO_3H_2$
WR-1065	$H_2N(CH_2)_3NHCH_2CH_2SH$
WR-2721	$H_2N(CH_2)_3NHCH_2CH_2SPO_3H_2$
Cysteamine (WR-347)	$HNHCH_2CH_2SH$
WR-151326	$CH_3NH(CH_2)_3NHCH_2CH_2CH_2SH$
WR-151327	$CH_3NH(CH_2)_3NHCH_2CH_2CH_2SPO_3H_2$
DTT	$HSCH_2CH(OH)CH(OH)CH_2SH$

Table II: Protection Factors for Cell Survival and for DNA Single- and Double-Strand Break Induction in CHO Cells Pretreated with Various Thiols for 30 min at 37°C.

Drug	PF[SF]	PF[SSB]	PF[DSB] (pH)
[illegible] mM WR 1065	[illegible]	[illegible]	[illegible] (7) [illegible] (9.6)
6 mM WR-255591	2.3	1.23	1.83 (7) 1.70 (9.6)
6 mM WR-151326	2.6-2.9	1.68	3.0 (7) 2.1 (9.6)
10 mM Cysteamine	2.8-3.1	1.82	2.5 (7) 2.0 (9.6)
25 mM DTT	2.3	1.68	1.8 (7) 1.65 (9.6)

Table III: Relationship Between PF[SF] and PF[SSB] for CHO Cells and for Mouse Tissues.

Drug	System	PF[SF]*	PF[SSB]	Ref.
WR-1065	CHO	2.0	1.27 ± 0.05#	1
WR-255591	CHO	2.?	1.23 ± 0.05#	?
WR-1065	Jejunum	2.0 (200)	1.13 ± 0.08* (400)	29
			1.24 ± 0.10# (400)	29
WR-2721	Jejunum	1.8 (400)	1.04 ± 0.07* (400)	29
WR-3689	Jejunum	1.5 (400)	1.0 (400)	33
		1.65 (800)	1.0 (800)	
WR-2721	Bone Marrow	1.5 (400)	1.6 (400)	34,16
		-2.2		

#PF[SSB]s were obtained using the ^{3}H-TdR assay where the cells were lysed with SDS rather than Sarkosyl for appropriate comparison with the CHO-cell data. *used Sarkosyl.

*All PF[SF] values are for clonogenic survival end points

APPENDIX: Manuscripts published or submitted which acknovledged either complete (**) or partial (*) support from Contract DAMD17-86-C-6105

1.* Murray, D. and Meyn, R. E., Differential repair of γ-ray-induced DNA strand breaks in vivo by various cellular subpopulations of mouse jejunal epithelium and bone marrow. Radiat. Res., 109, 153-164, 1987.

2.** Murray, D., vanAnkeren, S. C., Milas, L., and Meyn, R. E., The role of modification of DNA damage in the radioprotective action of aminothiols. Anticarcinogenesis and Radiation Protection, edited by P. A. Cerutti, O. F. Nygaard and M. G. Simic, (New York: Plenum Press) pp. 399-402, 1987.

3.** Murray, D., vanAnkeren, S. C., Milas, L., and Meyn, R. E., Radioprotective action of WR-1065 on radiation-induced DNA strand-breaks in cultured Chinese hamster ovary cells. Radiat. Res., 113, 155-170, 1988.

4.** Murray, D., Altschuler, E. M., Kerr, M. S., vanAnkeren, S. C., Meyn, R. E. and Milas, L., Radioprotection of cultured Chinese hamster ovary cells by WR-255591. Int. J. Radiat. Biol., 54, 269-283, 1988.

5.** Murray, D., vanAnkeren, S. C., Milas, L., and Meyn, R. E., A mechanistic approach to improving the efficacy of radioprotectors in radiotherapy. Progress in RadioOncology IV, edited by K-H Karcher, (Vienna: ICRO Press) pp. 169-172, 1988.

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7.** Murray, D., vanAnkeren, S. C., Milas, L., and Meyn, R. E., Radioprotective action of aminothiols in vitro and in vivo: Comparison between effects on DNA damage and cell survival. Pharmacol. Ther., 39, 151-153, 1988.

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10.* Travis E. L., and Murray, D., The clinical potential of normal tissue radioprotectors. Br. J. Radiol. in press, 1989.

11.** Murray, D., Altschuler, E. M., Hunter, N., and Milas, L., Protection by WR-3689 against γ-ray-induced intestinal damage: Comparison of the effect on DNA damage, clonogenic cell survival and mouse survival. Br. J. Cancer, submitted.

12.** Murray, D., Prager, A., and Milas, L., Effect of the aminothiols WR-1065 and WR-255591 on cell survival and DNA double-strand break induction in γ-irradiated cultured mammalian cells. Radiat. Res., submitted.

13.** Murray, D., Effect of thiols on DNA damage and repair. Critical Reviews in Thiol Biochemistry. In press.